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Stable Somatic Gene Expression in Mouse Lungs Following Electroporation-mediated Tol2 Transposon Delivery

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Gene delivery to the lung has rapidly progressed as an important method for studying various chronic lung diseases. Viral vectors, albeit highly efficient, are limited by the host immune response. Electroporation, a well-known non-viral method, can efficiently deliver genes to the lung, but is unable to induce stable gene expression. The Tol2 transposon is another non-viral method that can induce stable gene expression by reinserting its genes into the host genome. In this study, we combined electroporation and Tol2 transposons to obtain stable, high-level gene expression in the mouse lung. Tol2 transposon plasmids (pT2A-EGFP; Tol2, pCAGGS-TP; transposase) were optimized in vitro, and the electroporation procedure (pCAG-EGFP) was optimized in mouse lungs. After optimization, a combination of electroporation plus the Tol2 transposon was used in a comparative analysis with electroporation plus pCAG-EGFP. GFP expression levels were quantified and visualized on days 4 and 7 post-electroporation. We successfully reproduced the Tol2 transposon system in vitro and the electroporation procedure in vivo. We observed sustainable GFP expression using electroporation plus the Tol2 transposon on days 4 and 7, while electroporation plus pCAG-EGFP resulted in decreased GFP expression on day 7. We were able to induce high-level, stable gene expression in mouse lungs using a combination of electroporation and the Tol2 transposon. This represents a safer method for lung gene delivery that can be used as an alternative to viral vectors.

Lung gene delivery may provide a powerful tool for studies of lung diseases, such as pulmonary hypertension, chronic obstructive lung disease, cystic fibrosis, α1-antitrypsin deficiency, and lung cancer. Ideally, delivery methods must be safe, efficient, and confer stable gene expression. Viral vectors are widely used as a lung gene delivery system. These vectors are highly efficient and can induce stable gene expression in the lung (1-3). However, viral vectors induce an immunological response, resulting in the formation of antibodies against the vectors (4-7). Furthermore, generating high-quality viruses is expensive and laborious. Non-viral methods are associated with a lower risk of antibody formation, but have low efficiency (7-8). The latest non-viral methods demonstrate improved gene delivery efficiency.

Electroporation is one non-viral gene transfer method. It uses electrical fields to establish transient membrane pores that enable the entry of impermeable molecules, such as DNA, RNA, and proteins (9). Recent studies have shown that electroporation induces highly efficient gene expression in an in vivo mouse lung model (10-12). However, similar to other non-viral methods, gene delivery with electroporation assistance cannot induce stable gene expression in the mouse lung.

The Tol2 transposon is a mobile genetic element derived from medaka fish (Oryzias latipes) (13). It consists of gene insertion sequences that are excised by transposase and reinserted into the host genome to induce stable gene expression. Previous studies have shown that the Tol2 transposon is highly active in mammalian cells and could be applied to create transgenic mice (13-17). However, the Tol2 transposon has not yet been used for somatic gene delivery.

In this manuscript, we propose a new method to deliver somatic genes to mouse lungs using a combination of electroporation and the Tol2 transposon. Since electroporation is efficient and safe for use in mouse lungs, we hypothesize that the use of the Tol2 transposon followed by electroporation can induce stable gene expression in mouse lungs.

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MATERIAL AND METHODS

Animals

SV129 mice were housed in regular-sized cages in a controlled temperature and humidity, pathogen-free room with a 12-hour light cycle and free access to food and water. The animal experiments were conducted according to Kobe Pharmaceutical University Animal Care Guidelines.

Tol2 transposon plasmid

The Tol2 transposon-based pT2A-EGFP vector (5.5 kb) and Tol2 transposase expression vector (pCAGGS-TP) (6.9 kb) were kindly provided by Dr. Koichi Kawakami (National Institute of Genetics, Shizuoka, Japan) [15].

Cell transfection

Chinese hamster ovary cells (CHO-K1) were cultured in Ham nutrient mixture-F12 (Ham F12) with 10% fetal bovine serum. CHO-K1 cells were plated into 6-well plates for the transfection procedure. Approximately 1 µg of pT2A-EGFP and 1.2 µg of pCAGGS-TP plasmids were transfected into 3×10^5 cells using Lipofectamine LTX (Life Technologies, Carlsbad, CA, USA). GFP expression was observed in live cells on days 1, 7, 21, and 30 using an Olympus ix71 immunofluorescence microscope (Tokyo, Japan). Throughout the observation period, cells were maintained in normal conditions.

In vivo gene electroporation in the lung

Male SV129 mice (25–30 g) were anesthetized using sodium pentobarbital (50 mg/kg body weight). Fur around the back and chest was shaved with a chemical solution (Epilat Kracie). In a supine position, a 1-cm incision was made on the neck skin, and the sternohyoid muscles were separated to expose the trachea. A 30-gauge blunt needle was inserted between the cartilage rings. Mice were tilted to a 75° angle toward the standing position. Then, 100 µl of a solution containing 20 µg of pT2A-EGFP, 20 µg of pCAGGS-TP, 10 mM Tris (pH 8.0), and 0.9% NaCl was administered to the lung for approximately 3 seconds. For control animals, pCAG-EGFP was used instead of pT2A-EGFP and pCAGGS-TP. The needle was removed and the mice were allowed to recover their breathing pattern for 1 minute. Soon after, a 10-mm electrode (NEPAGENE CUY650P10, Chiba, Japan) was placed on the backside of mouse chests after smearing with the electroporation gel. Two series of 5-ms poring pulses and 5 series of 50-ms transfer pulses were applied using the NEPAGENE Nepa21 type 2 electroporator. For optimization, a serial voltage-dependent electroporation of 62, 80, 100, and 200 V was used. The incision was sutured and mice were placed on a warm pad (37°C). Mice were euthanized by pentobarbital overdose on days 4 and 7 post-electroporation. The lungs were removed and either directly snap-frozen in liquid nitrogen for GFP quantification or processed into fresh-frozen sections for histological analysis. The scheme for combination of electroporation and Tol2 transposon method were showed in Figure 1.

Histology

Immediately after euthanasia, the lungs were briefly perfused with 5 ml of 4% paraformaldehyde followed by 5 ml of phosphate-buffered saline. Mouse lungs were inflated with phosphate-buffered saline and directly embedded in a frozen block using optimum cutting temperature medium. Frozen blocks were sectioned (5 µm) and mounted on a slide glass. The sections were counterstained with DAPI and GFP expression was either directly visualized or double staining was performed with an anti-prosurfactant protein C antibody (PSC, ab90716; Abcam, Cambridge, UK). Samples were observed using an Olympus ix71 immunofluorescence microscope.

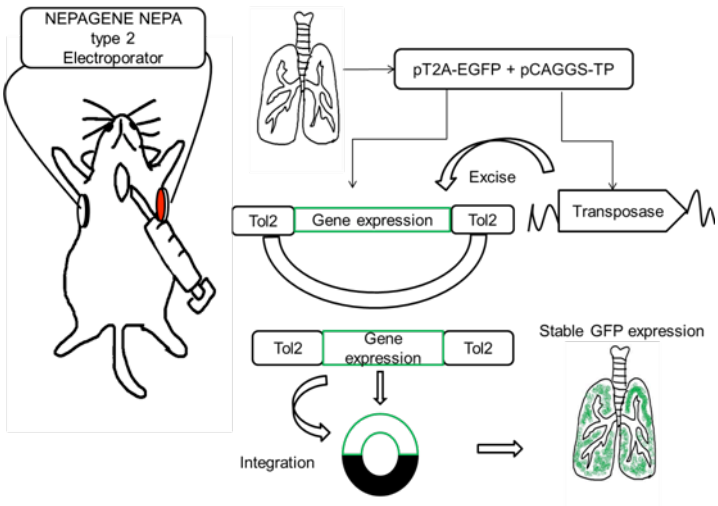


Figure 1. A scheme for the combination of electroporation and the Tol2 transposon. The Tol2 transposon-transposase plasmid solution (100 µl) was injected into the trachea and the solution was allowed to distribute to the lung for 1 minute by placing mice in the standing position. Shortly afterward, electrodes were placed on the side of the chest and the electroporation pulse was delivered. We expected that the Tol2 transposon could reinsert its gene into the lung genome and induce stable gene expression.

GFP quantification

GFP expression was quantified using the GFP Quantification Kit (BioVision, k815-100, Milpitas, CA, USA). Briefly, mouse lungs were homogenized with the assay buffer included in the kit. GFP expression was measured using Biofluoromark^{TM} and quantified according to the GFP Quantification Kit standard.

Statistical analysis

All measurements were analyzed using GraphPad Prism 6. Data are presented as means ± SEM. The data were analyzed using a one-way analysis of variance with the Sidak post-hoc analysis or Student's *t*-tests. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Tol2 transposon induced stable gene expression in CHO-K1 cells

Before using the Tol2 transposon in mouse lungs, we examined the ability of the pT2A-EGFP plasmid to induce stable gene expression in vitro. Our results showed that the pT2A-EGFP and pCAGGS-TP plasmids were able to induce stable GFP expression for up to 1 month (Figure 2).

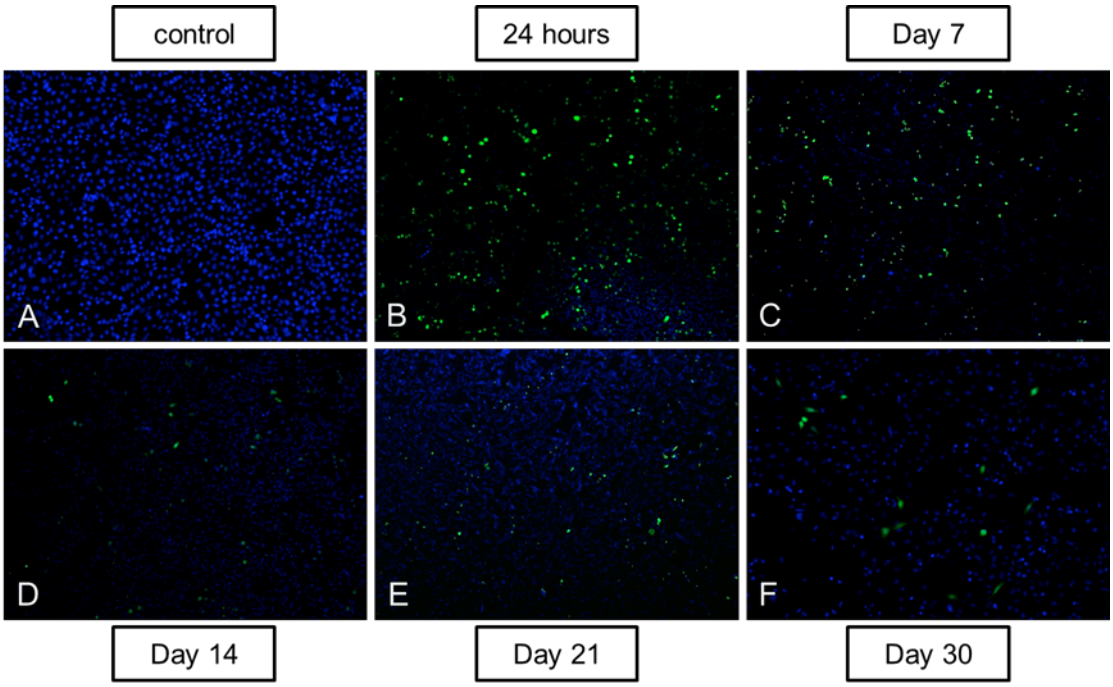


Figure 2. The Tol2 transposon induced stable gene expression in CHO-K1 cells. (A) A representative picture of CHO-K1 cells without plasmid addition. (B–F) Representative serial time observations of GFP expression in CHO-K1 cells transfected with Lipofectamine LTX, pT2A-EGFP, and pCAGGS-TP. DAPI (blue color), GFP (green color).

Electroporation for in vivo mouse lung gene transfer

We attempted to optimize the electroporation procedure using our model with a series of voltages. We observed that 100 V resulted in higher GFP expression than 62 V, 80 V, and 200 V (Figure 3 A–F). Based on our optimization, we used 100 V for our model.

Localization of GFP-expressed cells in the lung

In order to assess the GFP-expressed cell distribution in mouse lungs, we performed immunostaining with PSC, which is a marker for type 2 pneumocytes. We found that GFP-expressed cells partially co-expressed the PSC signal (Figure 3 G–H).

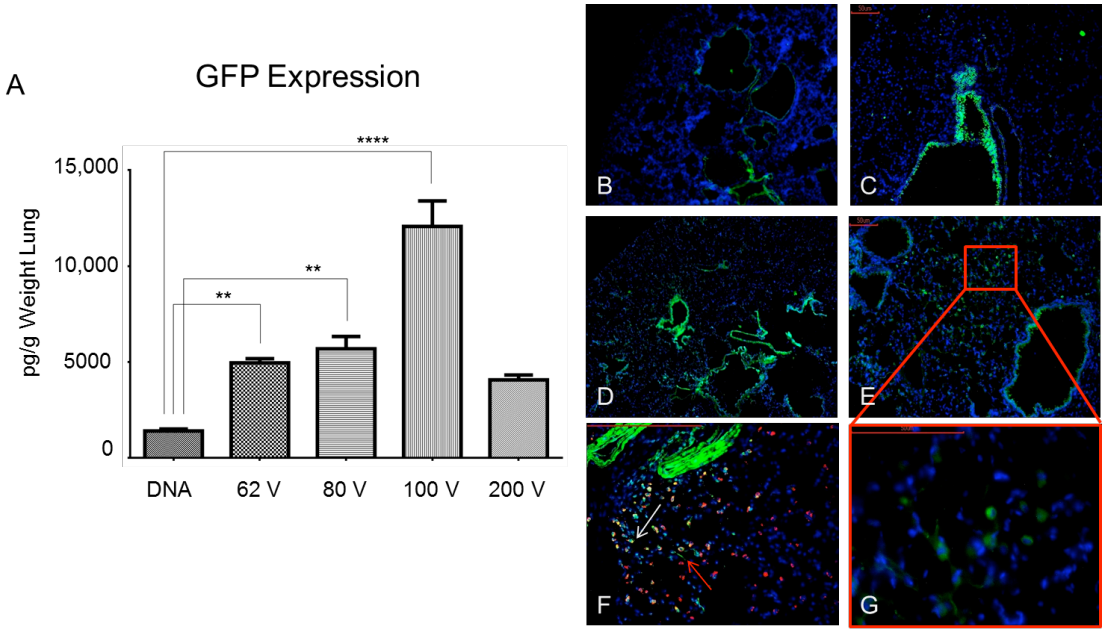
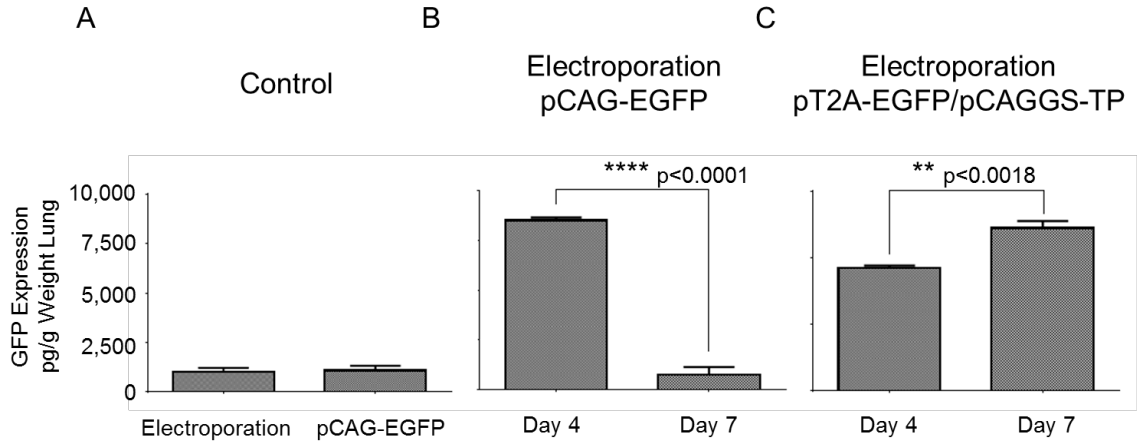


Figure 3. Electroporation-mediated gene delivery induced GFP expression in mice lung. (A) Quantification of lung GFP expression, n = 3 each group. (B-E) Representative serial Voltage immunofluorescence histology of GFP expression in the mouse lung after serial voltage treatment. Scale bars = 50 µm. (F) Representative picture of mouse lung double staining with anti-prosurfactant protein C antibody (PSC; red color) and GFP (green color). Scale bars = 50 µm. White arrowhead indicates co-localization of PSC staining with GFP expression (yellow color). Red arrowhead indicates GFP expression in pneumocyte type 1. Scale bars = 100 µm. (G) Magnified image of GFP expression in mouse lung for the 100-V group. Scale bars = 50 µm. Data are expressed as means ± SEM, ** p< 0.01, **** p< 0.0001. DAPI (blue color).

Electroporation-mediated Tol2 transposon induced stable gene expression in mouse lungs

We demonstrated that electroporation plus pCAG-EGFP could increase GFP expression, with a peak on day 4 and a decline on day 7 post-electroporation (Figure 4 B,E,H). The combination of electroporation, pT2A-EGFP, and pCAGGS-TP induced stable GFP expression throughout days 4 and 7 (Figure 4 C,F,I), without a detectable decline. We also observed GFP expression in the control group (Figure 4 A,D,G). This might have occurred due to auto-fluorescence, which is commonly encountered in mouse lung bronchi and bronchioles.



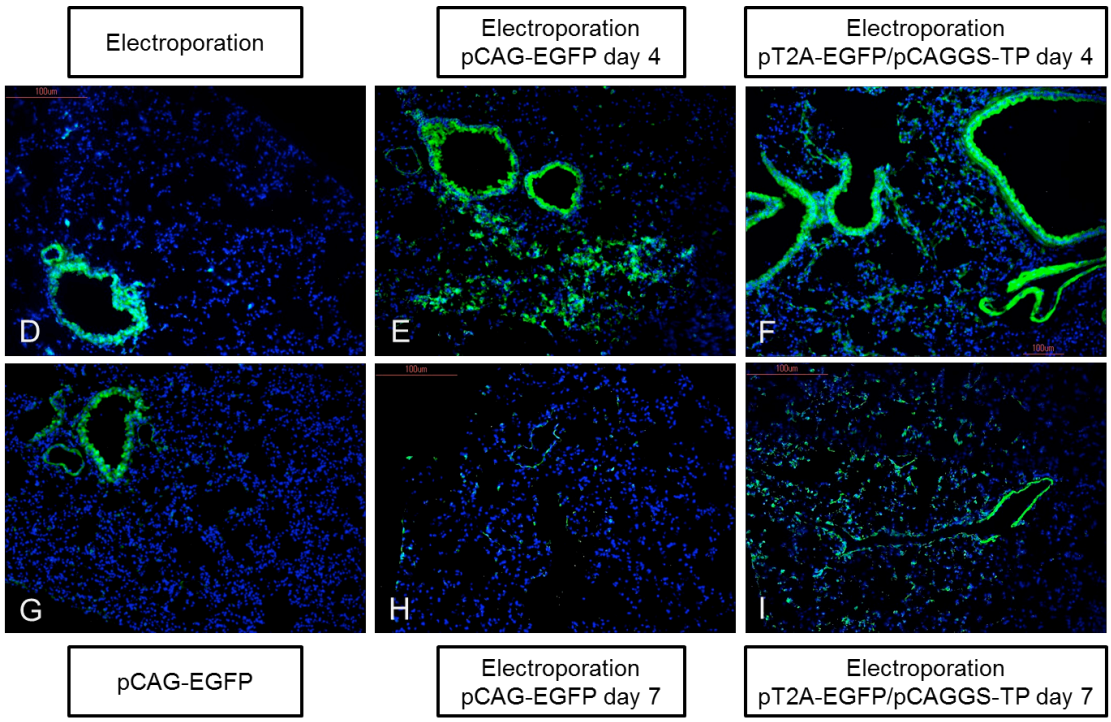


Figure 4. Electroporation-mediated Tol2 transposon induced stable gene expression in mouse lungs. (A) Quantification for GFP expression in the control group (electroporation, pCAG-EGFP). (B) Quantification for GFP expression after electroporation plus pCAG-EGFP. (C) Quantification for GFP expression after electroporation plus pT2A-EGFP/pCAGGS-TP. Representative picture of GFP expression after (D) electroporation, (E) electroporation+pCAG-EGFP day 4, (F) electroporation+pT2A-EGFP/pCAGGS-TP day 4, (G) pCAG-EGFP, (H) electroporation+pCAG-EGFP day 7, (I) electroporation+pT2A-EGFP/pCAGGS-TP day 7. Data are expressed as means ± SEM, ** $p<0.0018$, **** $p<0.0001$. DAPI (blue color), GFP (green color).

DISCUSSION

In the current study, we described a new method for stable gene delivery to the mouse lung. First, we verified that our Tol2 transposon can induce stable gene expression in CHO-K1 cells. Second, we observed that 100 V is optimal for electroporation gene delivery into mouse lungs. Third, we demonstrated that a combination of the Tol2 transposon and electroporation can induce stable gene expression in the mouse lung. These results proved that the combination of the Tol2 transposon and electroporation might be a useful somatic gene delivery method for the mouse lung.

To verify our method, we optimized our plasmids and the electroporation procedure. For plasmid verification, we observed stable GFP expression in CHO-K1 cells transfected with pT2A-EGFP and pCAGGS-TP, suggesting successful gene integration in the host genome (Figure 2) [17]. For the electroporation procedure, we discovered higher GFP expression in the lung in the 100-V group than in the other voltage groups (Figure 3 A–F). These electroporation results were consistent with previous studies that showed that electroporation can be used to efficiently deliver genes to mouse lungs (10-12). We also attempted to localize the distribution of GFP-transfected cells in our model. We found overlap between GFP cells and pneumocyte type 2 cells (Figure 3 G–H). Previous studies have shown that lung electroporation-mediated gene delivery can be used to transfect multiple cell types, including pneumocytes type 1 and 2 (10).

Previous reports have shown that, similar to other non-viral gene methods, electroporation-mediated gene delivery is unable to induce stable gene expression in the mouse lung (10-12). In concordance with these previous reports, we observed a decrease in GFP expression on day 7 in the electroporation plus pCAG-EGFP group. Various physical and biological barriers could hinder gene delivery to the lung. The mucociliary system in the bronchi impedes plasmid absorption and facilitates plasmid degradation. Furthermore, the non-integrating plasmid is degraded by DNAase over time (7). These limitations might be avoided by utilizing the ability of the transposon to reinsert its plasmid gene into the cell genome, inducing stable gene expression. Consistent with our expectation, we observed sustained GFP expression on day 7 using a combination of electroporation and the

Tol2 transposon, in contrast to the declining GFP expression observed in the electroporation and pCAG-EGFP group. These results suggest that the combination of electroporation and the Tol2 transposon can be used to induce stable gene delivery in mouse lungs. Unfortunately, we also found GFP expression in the control group. We believe that this GFP expression in the control group might reflect the green auto-fluorescent signal that is commonly observed for lung bronchi or bronchioles (18).

Thus, we were able to prove that with electroporation assistance, the Tol2 transposon could enter lung cells and integrate with the host genome, inducing stable gene expression. This result is in concordance with other transposon systems, such as Sleeping Beauty (SB) or piggyBac (PB) (19-21). Several studies have confirmed that Tol2 is highly efficient in most of cell lines compare with SB (22, 23). Previous studies have also shown that Tol2 demonstrated higher transposition efficiency in mouse germline compared with SB and PB (24). This high efficiency might be happen because Tol2 exhibits no overproduction inhibition, a phenomenon where transposition activity declines with increasing in transposase, which is demonstrated in SB and PB (22, 23). Furthermore, Tol2 has the highest DNA cargo capacity for up to 10 kb compared with 9.1 kb PB and 1.7 kb SB (15, 25, 26). These Tol2 transposon data are quite encouraging for future non-viral gene delivery systems that can provide sustained, stable gene expression.

Somatic gene delivery into the lung is considered as a novel method for various lung disorder treatment. Accumulated lung gene therapy clinical trials have been observed recently. Despite promising preliminary results, it is not yet widely acknowledged in the clinical setting due to unsatisfactory transfection efficiency (27, 28). Immunity responses to viral vector and lung barrier have been impeded gene delivery. In this case, we believe that Tol2 transposon system might be useful for clinical lung gene therapy in the future as an alternative to viral vectors by avoiding immunity response and induce stable gene expression. Further experiments are needed to elucidate the risk and possibilities of Tol2 transposon transfection for clinical lung gene therapy.

A major drawback of non-viral gene delivery methods is the short duration of gene expression. Using a combination of electroporation and the Tol2 transposon, we were able to induce high levels of stable gene expression in mouse lungs. This method may provide a solution for the limitations associated with general non-viral gene delivery. Since the Tol2 transposon possess a fairly large gene insertion capacity and highly efficient, it can be used as a safer method alternative to viral vectors for varied chronic lung disease studies.

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